

Use of the Post-Insertion Technique to Insert Peptide Ligands into Pre-Formed Stealth Liposomes with Retention of Binding Activity and Cytotoxicity

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Purpose: Simple methods for the large-scale manufacture of ligand-targeted liposomes will be needed if clinical trials are to proceed. We tested a recently developed technology for inserting peptide ligands into preformed Stealth liposomes. Antagonist G-targeted liposomes (PLG) were prepared and loaded with doxorubicin and their cellular association and cytotoxicity were evaluated using the human small cell lung cancer H69 cell line.

Methods: The hexapeptide antagonist G was covalently coupled via a thioether bond to the terminus of polyethylene glycol (PEG) in micelles formed from maleimide-derivatized poly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine followed by transfer into preformed liposomes during a one-step incubation. For cellular association, we used radiolabeled liposomes. Cytotoxicity was evaluated using the MTT *in vitro* proliferation assay.

Results: The postinsertion approach to the formation of peptide-targeted liposomes led to the production of PLG bearing a maximum of approximately 0.3 µg antagonist G/µmol phospholipid. These liposomes had increased cellular association to H69 cells relative to nontargeted liposomes and, when loaded with doxorubicin, they resulted in similar levels of cytotoxicity to those obtained by conventional coupling techniques.

Conclusions: The postinsertion technique is a simple, effective means for the production of biologically active peptide-targeted liposomes.

Key Words: antagonist G; polyethylene glycol; targeting; liposomes; doxorubicin; small cell lung cancer.

INTRODUCTION

Liposomes sterically stabilized with polyethylene glycol (M_r 2000) distearoylphosphatidylethanolamine conjugates (mPEG-DSPE), known as Stealth[®] liposomes (SL), have long circulation half-lives following intravenous injection (1). Entrapment of anticancer drugs in SL results in increased tumor accumulation and improved therapeutic efficacies, thus leading to the clinical approval of these novel liposome-based formulations (2). The development of methods for coupling cancer cell-specific ligands to a reactive terminus of the PEG chain has created new opportunities for the use of liposomes as homing devices for selective targeting of anticancer drugs to diseased cells. This strategy has been shown to improve the cytotoxicity and/or the therapeutic efficacy of encapsulated drugs over nontargeted liposomes in animal models of cancer (3).

We have recently shown that the growth factor antagonist H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH₂, named antagonist G (G), could be coupled to SL using conventional coupling techniques (SLG). Antagonist G is a broad-spectrum antagonist that competitively blocks the action of multiple neuropeptides (e.g., vasopressin, gastrin-releasing peptide, bradykinin) through its ability to bind to several receptors (through the residues D-Trp-N^{me}Phe-D-Trp-Leu) on the surface of small cell lung cancer (SCLC) cells (4). SLG selectively increased binding to, and internalization of liposomes by, the human SCLC H69 cell line relative to SL (5). Moreover, SLG exhibit long circulating times in blood, which is critical for *in vivo* tumor localization (6).

Preparation of SLG from liposomes that have coupling lipids incorporated in the bilayer during their formation involves several steps and a considerable length of time, and leaves some reactive functional groups on the inner leaflet of the lipid bilayer (7), which potentially can interfere with encapsulated materials. Simpler and more flexible methods for the large-scale manufacture of ligand-targeted liposomes will be needed if they are to proceed to clinical trials. Uster *et al.* demonstrated that mPEG-DSPE could be transferred rapidly from a micellar phase into the outer monolayer of preformed PEG-free liposomes in a time- and temperature-dependent manner, resulting in long-circulating liposomes (8). Maximum PEG insertion from the micelles into the liposomes was reached with 1 h incubations at 60°C. This postinsertion technique was extended to oligopeptide- (7), oligosaccharide- (7) and to monoclonal whole antibody-PEG-DSPE conjugates (9). In these latter two studies, transfer of ligands occurred even when the preformed liposomes already contained a few mol% of mPEG-DSPE. Ishida *et al.* showed that the postinsertion liposomes, made with monoclonal antibody anti-CD19-PEG-DSPE conjugates, resulted in a threefold increase in cellular association of the targeted liposomes to CD19⁺ human B cell lymphoma cells compared with nontargeted liposomes. This result was similar to that obtained with anti-CD19-targeted liposomes prepared by conventional coupling techniques (3,9).

In this work we investigated whether use of the postinsertion technology could be extended to the preparation of antagonist G-targeted liposomes (PLG). In addition, the cellular association and cytotoxicity of doxorubicin-loaded lipo-

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ABBREVIATIONS: PEG, polyethylene glycol; DSPE, distearoylphosphatidylethanolamine; mPEG-DSPE, methyl-terminated PEG (M_r 2000) linked via a carbamate bond to DSPE; Mal-PEG-DSPE, maleimide-terminated PEG-DSPE; HSPC; hydrogenated soy phosphatidylcholine; CHOL, cholesterol; SL, nontargeted sterically stabilized liposomes; antagonist G, H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH₂; SLG, sterically stabilized (pegylated) liposomes containing Mal-PEG-DSPE incorporated during liposome formation and with antagonist G covalently coupled to the PEG terminus; antagonist G-PEG-DSPE, micellar Mal-PEG-DSPE with antagonist G covalently attached to the PEG terminus; PLG, SL in which antagonist G-PEG-DSPE conjugates were inserted into the SL using the postinsertion approach; DXR, doxorubicin; SCLC, small cell lung cancer; [³H]-CHE, [1α,2α(n)-³H] cholesterylhexadecyl ether; PL, phospholipid; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); MES, 2-(N-morpholino)ethanesulfonic acid; IC₅₀, inhibitory concentration for 50% cell growth.

somes prepared by this technique were evaluated in the human SCLC H69 cell line.

MATERIALS AND METHODS

Materials

Antagonist G was synthesized by the Alberta Peptide Institute (Edmonton, AB). Fully hydrogenated soy phosphatidylcholine (HSPC) and methoxy (polyethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine (mPEG-DSPE) were generous gifts of ALZA Corp. (Mountain View, California). Cholesterol (CHOL) was purchased from Avanti Polar Lipids (Alabaster, Alabama). Maleimide-derivatized PEG₂₀₀₀-DSPE (Mal-PEG-DSPE) was custom synthesized by Shearwater Polymers, Inc. (Huntsville, Alabama). Sigma-cote, 2-iminothiolane, and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, Missouri). [$1\alpha,2\alpha(n)$ - ^3H] cholesterolhexadecyl ether, 1.48-2.22 TBq/mmol (^3H) -CHE) was purchased from Mandel Scientific (Guelph, Ontario). All other chemicals were of analytical grade purity.

Cell Line

The human SCLC cell line NCI-H69 (ATCC HTB-119) was purchased from the American Type Culture Collection. It was cultured in RPMI 1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO BRL, Grand Island, New York) and maintained at 37°C in a humidified incubator (90% humidity) in a 5% CO_2 atmosphere. Cells were maintained within their exponential growth phase.

Preparation of Liposomes

Small unilamellar liposomes composed of HSPC:CHOL:mPEG-DSPE at a 2:1:0.08 molar ratio, with or without 1 mol% of Mal-PEG-DSPE (total PEG of 5 mol% of phospholipid), were prepared as previously described (10). Size was determined by dynamic light scattering using a Brookhaven BI-90 particle size analyzer V7.2 (Brookhaven Instruments, Holtsville, New York). Liposomes containing doxorubicin, DXR (Faulding, Vaudreuil Inc., PQ), were prepared in 250 mM ammonium sulfate, pH 5.5, and loaded via an ammonium sulfate gradient, as previously described (11). The amount of encapsulated DXR was determined from its absorbance at 492 nm following dissolution in methanol. Phospholipid concentration was determined from either the specific activity of a [^3H] -CHE lipid tracer or by the Bartlett colorimetric assay (12).

Preparation of Antagonist G-Targeted Liposomes Either by the Postinsertion Method or by Conventional Techniques

Dried lipid films containing Mal-PEG-DSPE were hydrated at a concentration of 0.333 mM in 25 mM HEPES, 25 mM MES, and 140 mM NaCl (pH 6.5) with gentle agitation and heating at 65°C. Thiolated derivatives of antagonist G were obtained by reacting the peptide with 2-iminothiolane at a 1:4 molar ratio in 25 mM HEPES and 140 mM NaCl (pH

8.0) for 1 h at room temperature. At pH 8, 2-iminothiolane reacts with primary amines in a ring-opening reaction that regenerates the free sulfhydryl (13). The reaction is rapid and efficient (13–15) and the rate of hydrolysis was found to be negligible (13). Coupling of the thiolated antagonist G to the PEG terminus of Mal-PEG-DSPE micelles took place during an overnight incubation at room temperature, in siliconized-coated glassware (Sigmacote) in an inert N_2 atmosphere (16). Free maleimide groups were quenched with an excess of 2-mercaptoethanol for 30 min at room temperature. Antagonist G-coupled PEG-DSPE micelles were then concentrated in Centriscart I[®] tubes, size cut-off 5000 D (Sartorius AG, Goettingen) and then dialyzed, for the purpose of DXR loading, against a 250 mM ammonium sulfate solution overnight at 4°C in Pierce Slide-A-Lyzer Dialysis Cassettes (Pierce, Rockford, Illinois).

To prepare PLG, various ratios (1, 2, or 4 mol%) of antagonist G-coupled PEG-DSPE micelles to liposomal phospholipid (PL) were mixed with preformed liposomes for 1 h at 60°C. The resulting samples were then chromatographed on a Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) in either 25 mM HEPES, 140 mM NaCl (pH 7.4), or 100 mM sodium acetate, 70 mM NaCl (pH 5.5). The amount of antagonist G on the liposomes was determined by fluorimetry at $\lambda_{\text{ex}} = 288 \text{ nm}$ and $\lambda_{\text{em}} = 330 \text{ nm}$.

Preparation of SLG by a conventional coupling technique was carried out under the same experimental conditions described above. Instead of Mal-PEG-DSPE micelles, Mal-PEG-DSPE-grafted liposomes were incubated with the thiolated derivative of antagonist G overnight at room temperature, in pH 6.5 HEPES buffer. After reaction with an excess of 2-mercaptoethanol, the liposomes were chromatographed on a Sepharose CL-4B column in pH 7.4 HEPES buffer.

Cellular Association Studies

For cellular association studies, liposomes were labeled with [^3H] CHE. The term “cellular association” is used to indicate a combination of binding to the cell surface plus cellular internalization of the liposomes. SL, PLG, or SLG were incubated with 1×10^6 H69 cells for 1 h at 37°C, as previously described (3). The amount of [^3H] CHE-radiolabelled liposomes associated with cells was determined from scintillation counting. Liposomal cellular association was calculated from the specific activity of the lipid label [^3H] CHE in liposomes and was expressed as nmol of PL/ 10^6 cells.

In Vitro DXR Release Studies

All the DXR-containing liposomes (DXR-SL, DXR-SLG or DXR-PLG) were freshly prepared and free DXR, if any, was separated by elution over a Sephadex G-50 column (Pharmacia). DXR release was determined from 0–48 h at 37°C in either pH 7.4 HEPES buffer, cell culture medium, or 25% human plasma in pH 7.4 HEPES buffer. The percentage release of DXR was measured by fluorescence dequenching measured on a CytoFluor 2350 fluorimeter (Millipore, Bedford, MA) at $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$. Complete release (100% dequenching) of DXR was obtained by lysing the liposomes with 25 μl of 10% (v/v) Triton X-100 in distilled water per milliliter of liposome suspension.

Cytotoxicity Studies

The cytotoxicity of antagonist G-targeted liposomes containing DXR, prepared either by the postinsertion or the Mal-PEG-DSPE conventional coupling technique, was determined using the MTT cell proliferation assay. Briefly, H69 cells were seeded into 96-well plates at 8×10^4 cells/well and incubated for 2, 24, or 48 h at 37°C in an atmosphere of 95% humidity and 5% CO₂. At the end of the incubation the cells were gently washed twice with phosphate-buffered saline to remove unbound liposomes. The cells were then maintained in fresh medium at 37°C in an atmosphere of 95% humidity and 5% CO₂, for up to 5 days. Cell viability was then assessed as previously described (17). The results were expressed in terms of IC₅₀ (μ M of DXR) determined from the dose-response curves, and defined as the concentration of test agents that produced a 50% reduction in cell viability compared with drug-free control.

Statistical Analysis

The Student *t*-test was used to measure statistical significance between pairs of samples. Data was considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Antagonist G-targeted liposomes prepared by a conventional Mal-PEG-DSPE coupling technique (5,6) averaged 1 μ g of antagonist G/ μ mol of PL. The applicability of the postinsertion technique for the preparation of antagonist G-targeted liposomes was tested. The amount of antagonist G coupled to micelles was approximately 0.6 μ mol antagonist G/ μ mol of PEG-DSPE, which corresponded to a coupling efficiency of 60%. Preformed liposomes containing 4 mol% (with respect to phospholipid) mPEG-DSPE were incubated with 1, 2, or 4 mol% antagonist G-PEG-DSPE micelles, resulting in 0.15 ± 0.02 , 0.33 ± 0.08 , and 0.34 ± 0.15 μ g, respectively, of antagonist G/ μ mol PL inserted into the final liposomal preparations. Under these circumstances, 2–6% of antagonist G-PEG-DSPE in the micellar conjugates mixture was inserted into liposomes.

As expected, the cellular association of these samples with the H69 cell line increased in proportion to the amount of antagonist G conjugate incorporated into the preformed liposomes (Fig. 1). At incorporation levels in the range of 0.15–0.34 μ g antagonist G/ μ mol PL, the maximum cellular association of PLG (1–1.8 nmol PL/ 10^6 cells) was intermediate between that seen for SLG preparations containing either 0.3 (0.8 nmol PL/ 10^6 cells) or 1 μ g (2.8 nmol PL/ 10^6 cells) antagonist G/ μ mol PL (Fig. 1). Hence, it appears that PLG may have higher binding than SLG, at equivalent amounts of antagonist G, although the hydrophobicity of the peptide, combined with its small size, might lead to some nonspecific association of unconjugated peptide in SLG that does not contribute to binding. In contrast, when targeted liposomes are made by the postinsertion method, the peptide is first preconjugated to the large PEG-DSPE group and any unconjugated peptide is removed by dialysis. This should lead to a decrease in the nonspecific association of free peptide to the liposomes during the insertion step, resulting in higher apparent binding in the cellular assays.

It is important to point out that, although some mercap-

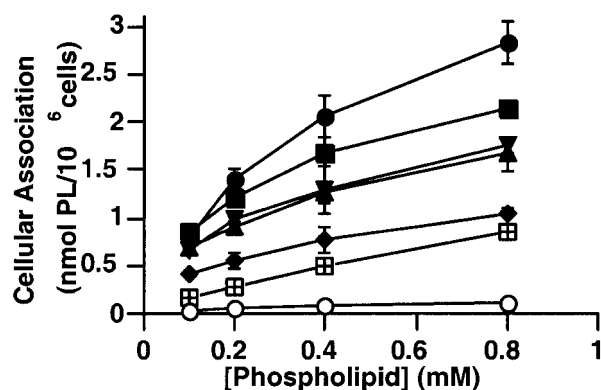


Fig. 1. Cellular association of [³H] CHE-labeled liposomes with H69 cells. Liposomes were incubated with 1×10^6 H69 cells for 1 h at 37°C at 0.1–0.8 mM PL/well. Nontargeted liposomes (—○—); targeted liposomes made by the postinsertion method containing 0.15 (—◆—), 0.33 (—▲—) or 0.34 (—▼—) μ g antagonist G/ μ mol PL. Targeted liposomes made by the Mal-PEG-DSPE method containing 0.3 (—◻—) or 1 μ g (—●—, —■—) antagonist G/ μ mol PL, where one of the samples (—■—) was concentrated and heated for 1 h at 60°C. Data was expressed as nmol of PL/ 10^6 cells. Each point is the mean of three samples, \pm SD, from one representative experiment.

toethanol-reacted Mal-PEG-DSPE conjugates are inserted into the outer monolayer of preformed liposomes, they are biologically inert. The resulting product does not influence the binding of liposomes at the cell level, which is evidenced by the binding data presented in Fig. 1. Nontargeted liposomes used in this experiment were originally made with the same amount of Mal-PEG-DSPE as targeted liposomes, and then quenched with an excess of 2-mercaptoethanol. The residual binding of these nontargeted liposomes (Fig. 1), is comparable to the cell binding obtained with nontargeted liposomes prepared with mPEG-DSPE (i.e., without Mal-PEG-DSPE, data not shown) and dramatically lower than that obtained with antagonist G-targeted liposomes. Others have confirmed the lack of cell binding of nontargeted liposomes made from mercaptoethanol-reacted Mal-PEG-DSPE conjugates (18,19).

In an attempt to clarify whether the heating step of the postinsertion methodology would affect the peptide binding, SLG containing 1 μ g antagonist G/ μ mol PL were concentrated and heated at 60°C for 1 h. Only a slight decrease in the cellular association was observed relative to nonheated liposomes containing the same amount of peptide (Fig. 1), suggesting that antagonist G was fairly stable to heating.

The transfer of antagonist G conjugates to performed, drug-loaded liposomes takes place above the phase transition temperature for HSPE, at 60°C; therefore, the tendency for the postinsertion method to cause release of the encapsulated DXR was elevated. Interestingly, a 1 h incubation at 60°C of DXR-loaded liposomes containing 4 mol% of mPEG-DSPE with antagonist G-PEG-DSPE micelles contributing a further 2 mol% of PEG, resulted in 87% of DXR leakage. Even when the content of mPEG-DSPE in preformed liposomes was increased to 9 mol%, 60% of drug leakage was observed at 1 h. Ishida *et al.* observed that little DXR leakage occurred when DXR-loaded liposomes (similar to those in this study) were heated at 60°C for 6 h, either alone or in the presence of IgG-PEG-DSPE micelles (9). These data suggest that hydro-

phobic antagonist G may cause some membrane perturbation, which could be either permanent or transient. Subsequently, experiments with DXR-loaded liposomes were performed by loading DXR after insertion of the antagonist G-PEG-DSPE conjugates. In these preparations, DXR release was evaluated in different media (Fig. 2). After 36 h at 37°C, regardless of the incubation media or the coupling method (SLG or PLG), the drug release was approximately the same as that observed for nontargeted liposomes. Thus, these data suggest that the membrane perturbation induced by insertion of antagonist G-PEG-DSPE conjugates may have been due to direct interaction of the hydrophobic peptide with the liposomal membranes during the transfer process. With completion of the insertion step, the peptide at the PEG terminus may be oriented away from the membrane surface, preventing further membrane disruption.

The size of the liposomes is a critical parameter for their *in vivo* application. Ideally, the average diameter of liposomes should be approximately 100 nm to minimize hepatosplenic uptake (20) and to ensure extravasation into diseased tissues such as solid tumors (21). The mean liposome diameter before and after insertion of antagonist G-PEG-DSPE was measured to determine if PLG met these requirements. For several experimental replicates the mean liposome diameter was only slightly increased (3–4 nm) by the insertion of antagonist G conjugates. Final diameters ranged from 94–99 nm with a polydispersion index of 0.060–0.100.

PLG exhibit apparent higher cellular association than SLG at similar peptide concentrations, so the cytotoxicity of DXR-loaded SLG vs. PLG was compared in the H69 cell line (Fig. 3). For liposomes containing similar amounts of peptide (0.3 µg/µmol PL), DXR-PLG were 18-fold more cytotoxic than DXR-SLG ($P < 0.001$) for a 2 h incubation. The magnitude of the difference decreased at longer incubation times, but remained statistically significant. There was a sixfold advantage for DXR-PLG at 24 h ($P < 0.001$) and twofold advantage at 48 h ($P < 0.01$). It is interesting to mention that although the cellular association for PLG coupled to 0.33 µg peptide/µmol PL was lower than that observed for SLG coupled to 1 µg peptide/µmol PL, both formulations medi-

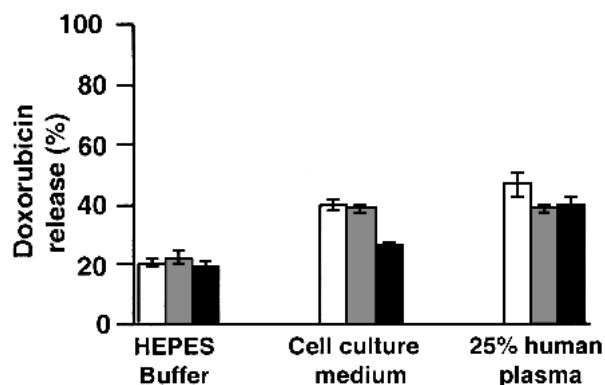


Fig. 2. Release of encapsulated self-quenched DXR from liposomes following their incubation in various media for 36 h at 37°C. DXR release from DXR-SL (□), DXR-PLG (▨), and DXR-SLG (■) was determined either in pH 7.4 HEPES buffer, cell culture medium, or in 25% human plasma in pH 7.4 HEPES buffer. Results were expressed as a percentage of total trapped DXR released. Each point is the mean of three to six samples, \pm SD, from one representative experiment.

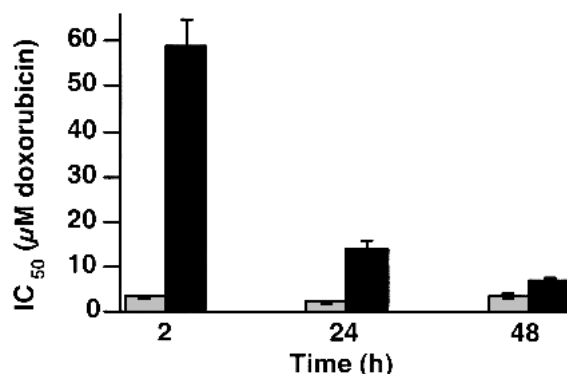


Fig. 3. Cytotoxicity of doxorubicin-loaded PLG vs. SLG against H69 cells. H69 cells (8×10^4 /well) were incubated with either DXR-PLG (▨) or DXR-SLG (■) containing 0.3 µg antagonist G/µmol PL. Incubations were carried out for 2, 24, and 48 h. Data is expressed as IC₅₀ in µM of DXR (mean \pm SD, of three to four independent experiments).

ated similar levels of cytotoxicity, according to previous data (5).

Overall, these data support the hypothesis that antagonist G on postinsertion targeted liposomes was either more available to interact with the receptors on the surface of the target cells and/or more efficiently internalized. For example, in SLG some of the peptide added during the coupling procedure may insert into the liposomal membrane through hydrophobic interactions and not couple to the PEG terminus. Control experiments in which SLG were incubated with liposomes containing Mal-PEG-DSPE under conditions in which no coupling could take place showed that approximately 16% of the added peptide was associated with the SLG in a non-specific manner. The fraction of the peptide that was not coupled to the PEG terminus in SLG would probably not participate in the binding process to cells. Cellular association represents binding + internalization, so the higher cellular association levels of PLG at similar amounts of coupled peptide may also be due to more efficient internalization of the PLG, leading to higher levels of DXR delivered intracellularly and higher levels of cytotoxicity.

In summary, we have shown that antagonist G could be coupled to Mal-PEG-DSPE micelles and the resulting peptide-PEG-DSPE conjugates could be transferred into pre-formed liposomes in a one-step incubation without significantly affecting liposome diameter. At similar levels of incorporated peptide, PLG had higher levels of cellular association than SLG, which resulted in greater cytotoxicity. Our data suggests that the postinsertion method of generating ligand-targeted liposomes may be an effective means of limiting non-specific absorption of ligand to liposomes when ligands with limited solubility, such as this hydrophobic peptide, are involved. The extension of the postinsertion technique to include peptide-PEG-DSPE conjugates confirms the flexibility of this approach to the preparation of targeted liposomes.

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REFERENCES

1. D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K. D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* **88**:11460–11464 (1991).
2. D. W. Northfelt, F. J. Martin, P. Working, P. A. Volberding, J. Russell, M. Newman, M. A. Amantea, and L. D. Kaplan. Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: pharmacokinetics, tumor localization, and safety in patients with AIDS-related Kaposi's sarcoma. *J. Clin. Pharmacol.* **36**:55–63 (1996).
3. D. E. Lopes de Menezes, L. M. Pilarski, and T. M. Allen. *In vitro* and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma. *Cancer Res.* **58**:3320–3330 (1998).
4. P. J. Woll and E. Rozengurt. A neuropeptide antagonist that inhibits the growth of small cell lung cancer *in vitro*. *Cancer Res.* **50**:3968–3973 (1990).
5. J. N. Moreira, C. B. Hansen, R. Gaspar, and T. M. Allen (eds.). Controlled Release Society, Inc. *In vitro* targeting of poly(ethylene glycol)-grafted liposomes by hexapeptide to a classical small cell lung cancer cell line. In *Proceedings of the 26th International Symposium on Controlled Release of Bioactive Materials*, June 20–25, Boston, Massachusetts, 1999, p. 6215.
6. J. N. Moreira, C. B. Hansen, R. Gaspar, and T. M. Allen (eds.). Organizing Committee of the IV Spanish-Portuguese Conference on Controlled Drug Delivery. Pharmacokinetic properties and biodistribution of hexapeptide-targeted Stealth® liposomes. In *Proc. IV Spanish-Portuguese Conference on Controlled Drug Delivery*, September 17–20, Vitoria, Spain, 2000, pp. 169–170.
7. S. Zalipsky, N. Mullah, J. A. Harding, J. Gittelman, L. Guo, and S. A. DeFrees. Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains. *Bioconjug. Chem.* **8**:111–118 (1997).
8. P. S. Uster, T. M. Allen, B. E. Daniel, C. J. Mendez, M. S. Newman, and G. Z. Zhu. Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged *in vivo* circulation time. *FEBS Lett.* **386**:243–246 (1996).
9. T. Ishida, D. L. Iden, and T. M. Allen. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. *FEBS Lett.* **460**:129–133 (1999).
10. F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail, and D. Papahadjopoulos. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta* **557**:9–23 (1979).
11. E. M. Bolotin, R. Cohen, L. K. Bar, N. Emanuel, S. Ninio, D. D. Lasic, and Y. Barenholz. Ammonium sulphate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes. *J. Liposome Res.* **4**:455–479 (1994).
12. G. R. Bartlett. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466–468 (1959).
13. G. T. Hermanson. Introduction of sulfhydryl residues (thiolation): modification of amines with 2-iminothiolane (Traut's reagent). In G. T. Hermanson, (ed.), *Bioconjugate Techniques*, Academic Press, San Diego, California, 1996, pp. 56–60.
14. R. Jue, J. M. Lambert, L. R. Pierce, and R. R. Traut. Addition of sulfhydryl groups to *Escherichia coli* ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate). *Biochemistry* **17**:5399–5406 (1978).
15. G. E. Means and R. E. Feeney. Chemical modifications of proteins: history and applications. *Bioconjug. Chem.* **1**:2–12 (1990).
16. F. J. Martin and D. Papahadjopoulos. Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting. *J. Biol. Chem.* **257**:286–288 (1982).
17. D. E. Lopes de Menezes, M. J. Kirchmeier, J. F. Gagne, L. M. Pilarski, and T. M. Allen. Cellular trafficking and cytotoxicity of anti-CD19-targeted liposomal doxorubicin in B lymphoma cells. *J. Liposome Res.* **9**:199–228 (1999).
18. D. Kirpotin, J. W. Park, K. Hong, S. Zalipsky, W. L. Li, P. Carter, C. C. Benz, and D. Papahadjopoulos. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. *Biochemistry* **36**:66–75 (1997).
19. D. L. Iden and T. M. Allen. *In vitro* and *in vivo* comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim. Biophys. Acta* **1513**:207–216 (2001).
20. D. C. Litzinger, A. M. Buiting, N. van Rooijen, and L. Huang. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. *Biochim. Biophys. Acta* **1190**:99–107 (1994).
21. O. Ishida, K. Maruyama, K. Sasaki, and M. Iwatsuru. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. *Int. J. Pharm.* **190**:49–56 (1999).